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TITLE: Regulation of Ubiquitin Mediated Proteolysis of G1 Cyclins
and the CDK Inhibitor p27 by the Cullin Gene Family in
Normal and Tumorigenic Human Breast Cells

PRINCIPAL INVESTIGATOR: Jennifer Michel
Yue Xiong, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
Chapel Hill, North Carolina 27599-1350

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FOREWORD

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Jennifer J. Michel
Lineberger Comprehensive Cancer Center
Manning Dr. and Mason Farm Rd., CB#7295
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7295

July 2000 Annual Summary Report for Award Number DAMD17-98-1-8221

INTRODUCTION

Sequential activation and inactivation of cyclin dependent kinases (CDK) regulate eukaryotic cell cycle transitions. The periodicity of CDK activity provides a molecular basis for unidirectional cell cycle progression and is controlled, in part, by the ubiquitin-mediated proteolysis of both cyclins and CDK inhibitors. Deregulated expression of G1 CDK inhibitors and G1 cyclins have been directly linked to breast cancer development. The mechanisms regulating the ubiquitination of these two families of proteins constitute essential components of G1 cell cycle control in mammalian cells and are poorly understood at present. These proteins, cyclin D, cyclin E, p27 and p57, are believed to be degraded in a phosphorylation-dependent manner by the ubiquitin-mediated proteasome pathway. This proteolysis pathway is composed of a cascade of enzymes, E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) that catalyze the attachment of ubiquitin to substrates via terminal isopeptide bonds to form polyubiquitinated conjugates. These species of proteins are rapidly recognized and degraded by the 26S proteasome. The E3 ubiquitin ligase is functionally defined to contain two separate activities: the ubiquitin ligase activity to catalyze isopeptide bond formation and the substrate targeting activity. Thus, two issues critical to our understanding of the regulation of protein turnover are how E3 ligases target proteins for ubiquitination and how they themselves are regulated. Cullin-1 represents a multigene family of proteins and has been found to be a component of E3 ligases. In budding yeast, the gene product of CDC53 partners with ROC1 to form an E3 ligase, and association with the SKP1 protein and F-box containing proteins mediates the ubiquitin-dependent degradation of a G1 cyclin and a G1 CDK inhibitor (called the SCF pathway). Hence, Cdc53p and Roc1p are responsible for regulating the G1-to-S transition in yeast. The purpose of this line of research is to determine how G1 cyclins and G1 CDK inhibitors are regulated by proteolysis during the mammalian cell division cycle and to determine in what other ways Cullin/ROC E3 ligases may contribute to proper cell cycle progression. Thus, this research could uncover previously unknown molecular aberrations associated with breast cancer development, potentially leading to improved diagnostic, prognostic, and therapeutic strategies.

BODY

The following is a description of work accomplished pertaining to the Statement of Work for months 13-24.

According to the approved Statement of Work, Task 2 was to identify cullin members that may interact with cyclin D, cyclin E and p27. Months 9-14 were to be used performing the yeast-two-hybrid assay using cullin members as bait and cyclin D, cyclin E and p27 as prey. Months 15-19 were to be spent characterizing anti-CUL-3 and anti-CUL-4 antibodies generated. Months 20-22 were to be used to construct pCMV-HA-cyclin E(T380A) and pCMV-HA-p27(T187A). Months 23-30 were to be spent identifying cyclin E-, cyclin D-, and p27- interacting cullins using IP-Western techniques. The following is a description of results pertaining to the relevant timeline for Task 2.

As described in the July 1999 report, direct interaction of cullins with cyclins D and E or the CDK inhibitor p27 has not been detected. Expression of human cyclin E in yeast is toxic which complicates the use of the yeast-two-hybrid system for this purpose. In yeast-two-hybrid screens done in the lab using p27 and cyclin D as bait, no cullins were detected to interact. These negative results could be explained by the following reasons. First, phosphorylation of substrates has been found to play a major role in the recognition of and targeting to the Cullin-1/ROC E3 ligase. Thus, cullins may not interact with a substrate, such as p27, in the yeast-two-hybrid system because it is not properly modified. Second, cullins may or may not be interacting directly with substrate. Thus far, in the case of Cullin-1, a SKP-1 and F-box pathway is required to interact with substrate. It remains to be seen how other cullins interact with substrate, though the evidence points to a SKP-1 independent targeting pathway. In coupled transfection-IP western experiments done in our laboratory, cyclin Ds have been detected to specifically interact with ROC1 and ROC2. Since ROC proteins interact tightly with cullins, it follows that cyclin Ds could be found in a complex with cullins. Whether the cyclin Ds are substrates of the Cullin/ROC ligases or if cyclin Ds provide a regulatory function specifically for ROC proteins remains to be seen.

The characterization of the Cullin-3 and Cullin-4 antibodies is complete. As reported in the July 1999 summary, the original antibodies that were made against C-terminal peptides for Cullin-3 and Cullin-4 were not useful. Thus, we generated a new antibody to Cullin-3 using an N-terminal peptide. This antibody has been characterized, and it works very well. It is capable of western blotting Cullin-3 proteins and immunoprecipitating Cullin-3 protein. Thus, this antibody will be useful for future experiments. The generation of a Cullin-4 antibody using an N-terminal peptide was not feasible as the sequence of the extreme N-terminus was not available. Therefore, we do not have a viable antibody against Cullin-4.

The pCMV-HA-cyclin E(T380A) and pCMV-HA-p27(T187A) plasmids have been successfully constructed and are available for use in experiments. Both plasmids have been used for transfection and express their respective gene products well in several cell lines used. Reciprocal IP-Western experiments done using the cullin-3, cyclin E, cyclin D, and p27 antibodies available in the laboratory, using the above described plasmids for transfection, and using proteasome inhibitors have not yielded any detectable interactions between cullin-3 and cyclin E, cyclin D, or p27. It is possible that these lines of research have not yielded positive

result due to the limitations of the antibody reagents, which may be incapable of co-immunoprecipitating the respective associated cullins, cyclins, or CDK inhibitors. The experiments described in Task 2, though unsuccessful, are complete.

In an effort to further expound upon the studies initiated in Task2 and to better understand the putative contributions of other cullin family members in cell cycle control, I moved to the yeast system where two cullin homologues, Cullin B and Cullin C, exist. Using an established ubiquitin ligase assay, I was able to determine that both Cullin B and Cullin C have an associated ubiquitin ligase activity and, thus, most likely function as E3 ligases like the members of the mammalian cullin family. I was able to show further, using yeast genetic analysis and the yeast-two-hybrid system, that Cullin B and Cullin C are functionally distinct from Cdc53. To gain a better understanding of their individual contributions to cell cycle regulation, Cullin B and Cullin C were individually deleted from yeast, and the strains were analyzed. Deletion of Cullin B did not appear to affect the cells under normal conditions. However, deletion of Cullin C resulted in a slower growth rate. Further analysis by Hoechst staining (which stains the DNA) and microscopic inspection demonstrated a population of large budded cells with unorganized DNA and a second population of budding cells where the DNA was positioned midway in the bud neck, indicative of a mid-anaphase delay. The mid-anaphase defect, due to the lack of Cullin C function, was confirmed when ectopic expression of Cullin C but not Cullin B, CDC53 or vector alone rescued the mid-anaphase delay. Ectopic expression of human cullins also did not rescue the mid-anaphase delay, obscuring these efforts to determine if any of the human cullins was a functional homologue of Cullin C.

A similar mid-anaphase delay phenotype dependent upon the Rad9 DNA damage gene has been described. In an attempt to determine if the mid-anaphase delay induced by Cullin C deletion was also dependent upon Rad9 function, I constructed a yeast strain that was double mutant for Cul-C and Rad9. This double mutant rescued the mid-anaphase delay by 10-fold, though Cullin C does not seem to play a role in the UV DNA damage pathway described for Rad9. However, I have been able to provide evidence that Cullin C may play a role in facilitating mitosis though its exact function is not clear. Notably, this is the first evidence that another ubiquitin ligase other than the APC functions in mitosis. Furthermore, studies of the yeast cullin homologues have benefited our knowledge of this multigene family, providing evidence for multiple cullins in cell cycle control.

Additionally, we have completed an extensive characterization of the ROC family proteins (described in the July 1999 annual report) that has been published in the journal *Oncogene* in 1999 (see attached reprint). Briefly, we were able to demonstrate that ROC1, ROC2, and APC11 genes are induced by mitogenic stimulation, but that the level of these proteins are constant during the cell cycle. Furthermore while endogenous ROC1 and APC11 proteins are stable, ectopically expressed ROC1, ROC2 and APC11 are short-lived proteins, sensitive to proteasome inhibitors. We have also been able to demonstrate that disruption of ROC1-Cullin association decreases the stability of ROC1 and abolishes its associated ubiquitin ligase activity, providing evidence for a potential mechanism by which ROC-Cullin ligases could be regulated.

As this and the previous report describe, we have made much progress in understanding the role of the cullin family in regulating the proteolysis of cell cycle proteins. As we continue our work in this field, we hope to define the mechanisms targeting mammalian G1 cyclins and G1 CDK inhibitors for regulated proteolysis, the mechanisms regulating the Cullin/ROC E3 ligases, and the extent of the roles of the Cullin/ROC E3 ligase family in cell cycle control. Understanding these mechanism could aid in understanding how some molecular aberrations occur in breast cancer development, and it could allow us to contribute to improved diagnostic, prognostic, and therapeutic strategies for this disease.

Please note that the remaining year of grant award DAMD17-98-1-8221 will be transferred to Joseph McCarville, a graduate student in the laboratory of Dr. Yue Xiong, as of July 1, 2000.

APPENDIX A

Key Research Accomplishments

- Completion of Task 2 experiments
- Additional characterization of the ROC family
- Characterization of yeast homologues, Cullin B and Cullin C
- Implication of a potential role for Cullin C in proper mitotic control

List of Reportable Outcomes

Manuscripts

Ohta, T., Michel, J.J., and Xiong, Y. (1999). Association with cullin partners protects ROC proteins from proteasome-dependent degradation. *Oncogene* 18:6758-6766.

Michel, J.J., McCarville, J., and Xiong, Y. (2000). Yeast Cullin C may contribute to proper mitotic progression. Manuscript in preparation.

Abstracts

Michel, J.J. and Xiong, Y. Cell cycle regulation by ubiquitin mediated proteolysis: Roles of Cullin-ROC ubiquitin ligases. Era of Hope Department of Defense Breast Cancer Research Program Meeting, June 8-11, 2000. Hilton Atlanta and Towers, Atlanta, GA.

Presentations

Era of Hope Department of Defense Breast Cancer Research Program Meeting, Hilton Atlanta and Towers, Atlanta, GA, June 11, 2000

Degrees Obtained Supported by this Award

Ph.D. from the Curriculum in Genetics and Molecular Biology awarded from the University of North Carolina at Chapel Hill, Chapel Hill, NC in May 2000.

Employment Applied for and Received Based on Training Supported by this Award

Postdoctoral position with Dr. John Scott of the Oregon Health Sciences University applied for, received, and to begin July 5, 2000.

Association with cullin partners protects ROC proteins from proteasome-dependent degradation

Tomohiko Ohta^{1,5}, Jennifer J Michel² and Yue Xiong^{*1,2,3,4}

¹Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599-7295, USA; ²Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599-7295, USA; ³Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599-7295, USA; ⁴Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599-7295, USA

Cullin 1/CDC53 represents a multigene family and has been linked to the ubiquitin-mediated proteolysis of several different proteins. We recently identified two closely related RING finger proteins, ROC1 and ROC2, that share considerable sequence similarity to an APC subunit, APC11, and demonstrated ROC1 as an essential subunit of CUL1 and CDC53 ubiquitin ligases. We report here that the expression of ROC1, ROC2 and APC11 genes are induced by mitogens and remain constant during the cell cycle. Unlike other subunits of SCF and APC E3 ligases, ectopically expressed ROC family proteins are degraded by a proteasome-inhibitor sensitive pathway and are stabilized by associating with cullins. Mutations at the conserved Phe79 and His80 residues in the RING finger of ROC1 diminish its binding with cullins, resulting in a loss of cullin protection and ubiquitin ligase activity. These results suggest a potential mechanism for regulating the activity of ROC-cullin ligases through complex assembly and ROC/APC11 subunit ubiquitination.

Keywords: ROC family; cullins; proteasome-dependent degradation

Introduction

The ubiquitin-proteasome pathway plays a key role in regulating the abundance of a broad range of cellular proteins. Through a cascade of enzymes involving ubiquitin activating (E1), conjugating (E2) and ligating (E3) activities, this pathway catalyzes the formation of polyubiquitin chains onto substrate proteins via isopeptide bonds. Polyubiquitinated substrates are then rapidly detected and degraded by the 26S proteasome (Jentsch, 1992; Hochstrasser, 1996; Herschko, 1997). E1 and E2 both represent structurally related proteins and are relatively well characterized biochemically. The E3 ubiquitin ligases, however, are generally defined to contain both a ubiquitin ligase activity and a substrate targeting function, and they comprise a potentially large number of diverged multi-subunit protein complexes (King *et al.*, 1996; Patton *et al.*,

1998). Elucidating the molecular nature and regulation of E3s have become critical issues to our understanding of regulated proteolysis and is currently under intensive investigation.

The three best characterized E3 activities are HECT (homologous to E6AP carboxy terminus) domain proteins represented by E6-AP (Huibregtse *et al.*, 1995), the anaphase-promoting complex (APC or cyclosome) that consists of at least 12 subunits and is required for both entry into anaphase as well as exit from mitosis (King *et al.*, 1995; Sudakin *et al.*, 1995; Yu *et al.*, 1998; Zachariae *et al.*, 1998), and the SCF that consists of SKP1, CDC53/cullin-1 and a distinct F box protein that functions to ubiquitinate many phosphorylated substrate proteins (Bai *et al.*, 1996; Skowyra *et al.*, 1997; Feldman *et al.*, 1997; Verma *et al.*, 1997). Mutant Cdc53 failed to separate the spindle pole bodies, to initiate DNA replication (Mathias *et al.*, 1996) and to degrade the G1 CDK inhibitor, p40^{SK1} (Schwob *et al.*, 1994; Willems *et al.*, 1996; Bai *et al.*, 1996). Cullin 1 was initially identified from a screen for mutants with excess postembryonic cell divisions in *C. elegans* whose loss of function caused hyperplasia of all tissues as a result of the failure to properly exit from the cell cycle (Kipreos *et al.*, 1996). CUL1/CDC53 represents an evolutionarily conserved multigene family that includes at least seven members in *C. elegans*, six in humans, and three in budding yeast including Cdc53p (Kipreos *et al.*, 1996; Mathias *et al.*, 1996). Like yeast CDC53, human cullin 1 directly binds to SKP1 to form a multi-subunit complex with an F box protein, β -TrCP, to mediate the degradation of phosphorylated I κ B α and β -catenin (Yaron *et al.*, 1998); and recently reviewed in Maniatis (1999); Laney and Hochstrasser (1999), indicating that a similar mechanism might be employed for the function of mammalian CUL1. A subunit of the mitotic APC E3 complex, APC2, was found to contain limited sequence similarity to CDC53/cullins (Zachariae *et al.*, 1998; Yu *et al.*, 1998). These findings, together with the fact that there are no obvious structural similarities between other components of the SCF and APC, underscore an important and conserved role for cullin proteins in ubiquitin-mediated proteolysis, possibly as an intrinsic subunit of ubiquitin ligases.

Very recently, a family of closely related RING finger proteins, represented by ROC1 (regulator of cullins, (Ohta *et al.*, 1999; Tan *et al.*, 1999)), Rbx1 (RING-box protein, (Kamura *et al.*, 1999; Skowyra *et al.*, 1999)), or Hrt1 (Seol *et al.*, 1999) was identified that shares a high degree of sequence similarity to another APC subunit, APC11. ROC1/Rbx1/Hrt1

*Correspondence: Y Xiong, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599-7295, USA

⁵Current address: Department of Surgery, St Marianna University School of Medicine, Kawasaki, 216 Japan

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functions as an essential subunit of the mammalian CUL1 ubiquitin ligase to catalyze ubiquitination of phosphorylated I κ B α and as an essential subunit of yeast Cdc53p to catalyze ubiquitination of the G1 cyclin Cln2 and CDK inhibitor Sic1 (Ohta *et al.*, 1999; Tan *et al.*, 1999; Kamura *et al.*, 1999; Skowrya *et al.*, 1999; Seol *et al.*, 1999). Deficiency of yeast ROC1 can be functionally rescued by mammalian ROC1 and ROC2, but not yeast APC11 (Ohta *et al.*, 1999; Kamura *et al.*, 1999; Seol *et al.*, 1999), demonstrating an evolutionary conservation and functional specificity for the ROC/Rbx gene family. Mutational analysis and biochemical reconstitution experiments provide evidence that ROC1 and cullin 1 form heterodimeric ligases (Ohta *et al.*, 1999; Tan *et al.*, 1999). ROC1 (and ROC2) commonly interact with all cullins while APC11 specifically interacts with cullin-related APC2 (Ohta *et al.*, 1999), suggesting the existence of a potentially large number of heterodimeric ubiquitin ligases. How the ROC-cullin family of ubiquitin ligases are regulated is unclear. This report provides evidence that a potential mechanism to regulate this family of ubiquitin ligases is ROC/APC11-cullin/APC2 complex assembly and ROC/APC11 subunit degradation.

Results

Expression of ROC1, ROC2 and APC11 genes are induced by mitogenic stimulation

ROC1/ROC2 and APC11 selectively interact with cullins and the cullin-related APC2 to constitute dimeric ubiquitin ligases that function during interphase and mitosis, respectively. To gain insight into the

regulation of ROC/APC11, we examined the expression of these genes in response to mitogenic stimulation and during the first cell cycle. Normal human fibroblasts were arrested in quiescence by serum starvation and released into the cell cycle by serum addition, as monitored by FACS analysis (Figure 1a). The steady state levels of ROC1, ROC2 and APC11 mRNA at different time points after release into the cell cycle were determined by Northern blotting. As another control to monitor cell cycle progression, the blot was re-probed with a probe derived from the p21 CDK inhibitor whose mRNA is expressed at a high level in quiescent cells, further accumulates immediately following mitogenic stimulation and declines as cells move toward the G1/S boundary (Li *et al.*, 1994). Both ROC1 and ROC2 mRNAs as well as CUL1 mRNA are expressed at readily detectable levels in quiescent and G1 cells, become evidently induced at the G1-to-S transition following mitogenic stimulation, and remain relatively constant throughout the cell cycle (Figure 1b).

To determine whether the steady state levels of ROC/APC11 family proteins are accordingly induced by mitogenic stimulation, total cell lysates were prepared from quiescent and serum stimulated NHF cells and immunoblotted with antibodies to either ROC1 or APC11. To monitor cell cycle progression the same blot was also probed with antibodies specific to D cyclins, which are induced during early G1, and an antibody specific to cyclin B, which is induced in S phase. Consistent with the Northern analysis, the steady state levels of both ROC1 and APC11 proteins are low in quiescent cells and are induced by serum addition with kinetics that follow their mRNA inductions (Figure 1c). The level of E2 CDC34 protein

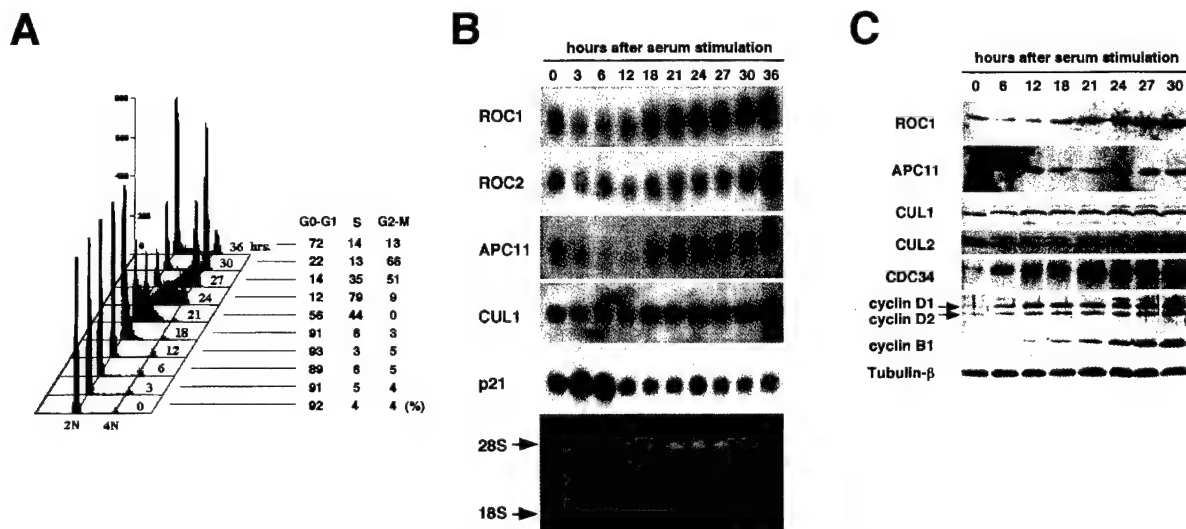


Figure 1 Mitogenic induction of ROC1, ROC2 and APC11. (a) Normal human foreskin diploid fibroblasts (strain NHF1) were arrested in G0 by serum deprivation and released from quiescence by serum stimulation. Exit from quiescence and progression through the cell cycle following serum stimulation was monitored by FACS analysis. (b) Total RNA was prepared from cells at different time points after stimulation by serum readdition. 5 μ g of RNA from each preparation were loaded onto a 1.5% agarose gel, and equal RNA loading was further confirmed by ethidium bromide staining (lower panel). Resolved RNA was transferred to a nitrocellulose filter, and the blot was hybridized with a series of probes derived from the coding region of the indicated human cDNAs. As an alternative control for monitoring cell cycle progression, the same blot was also hybridized with a probe corresponding to the G1 accumulated CDK inhibitor p21 (Li *et al.*, 1994). (c) Fifty μ g of total lysates obtained from the synchronized cells described in (a) were resolved by SDS-PAGE. The steady state levels of the different proteins were determined by direct immunoblotting.

was undetectably low in quiescent cells and was also induced by mitogenic stimulation. The level of CUL1 protein was readily detectable in quiescent cells and underwent minimal changes following mitogenic stimulation. Taken together with our previous finding that ROC1-CUL1 complexes may be activated differently by various E2s (Ohta *et al.*, 1999), these observations suggest that though the CUL1-ROC1 complex may be assembled in quiescent cells, CDC34-dependent ROC1-cullin 1 ubiquitin ligase activity would be absent until activated by mitogens.

The level of ROC1, ROC2 and APC11 proteins are constant during the cell cycle

To determine whether the steady state levels of ROC/APC11 proteins oscillate during the normal cell cycle, we arrested the cells at the G1/S boundary by a double

thymidine block. HeLa cells were chosen for this study as they can be arrested and then released to synchronously progress through the cell cycle without entering a quiescent state like normal fibroblasts. Progression of HeLa cells through S, G2/M and into the next G1 phase after release from the thymidine block was monitored by FACS analysis (Figure 2a). It was also confirmed by determining the level of cyclin B1 protein, which accumulates during S and G2 and is destroyed abruptly at the M/G1 transition (Figure 2b, bottom panel). The steady state levels of ROC1, APC11, CUL1 and CDC34 remained relatively constant in HeLa cells during the progression through S, G2/M and G1 phases of the cell cycle (Figure 2b). Consistent with this finding, the ubiquitin ligase activities of both ROC1 (Figure 2c) and CUL1 (Figure 2d) did not fluctuate significantly during the same periods of the cell cycle. There is a slight increase

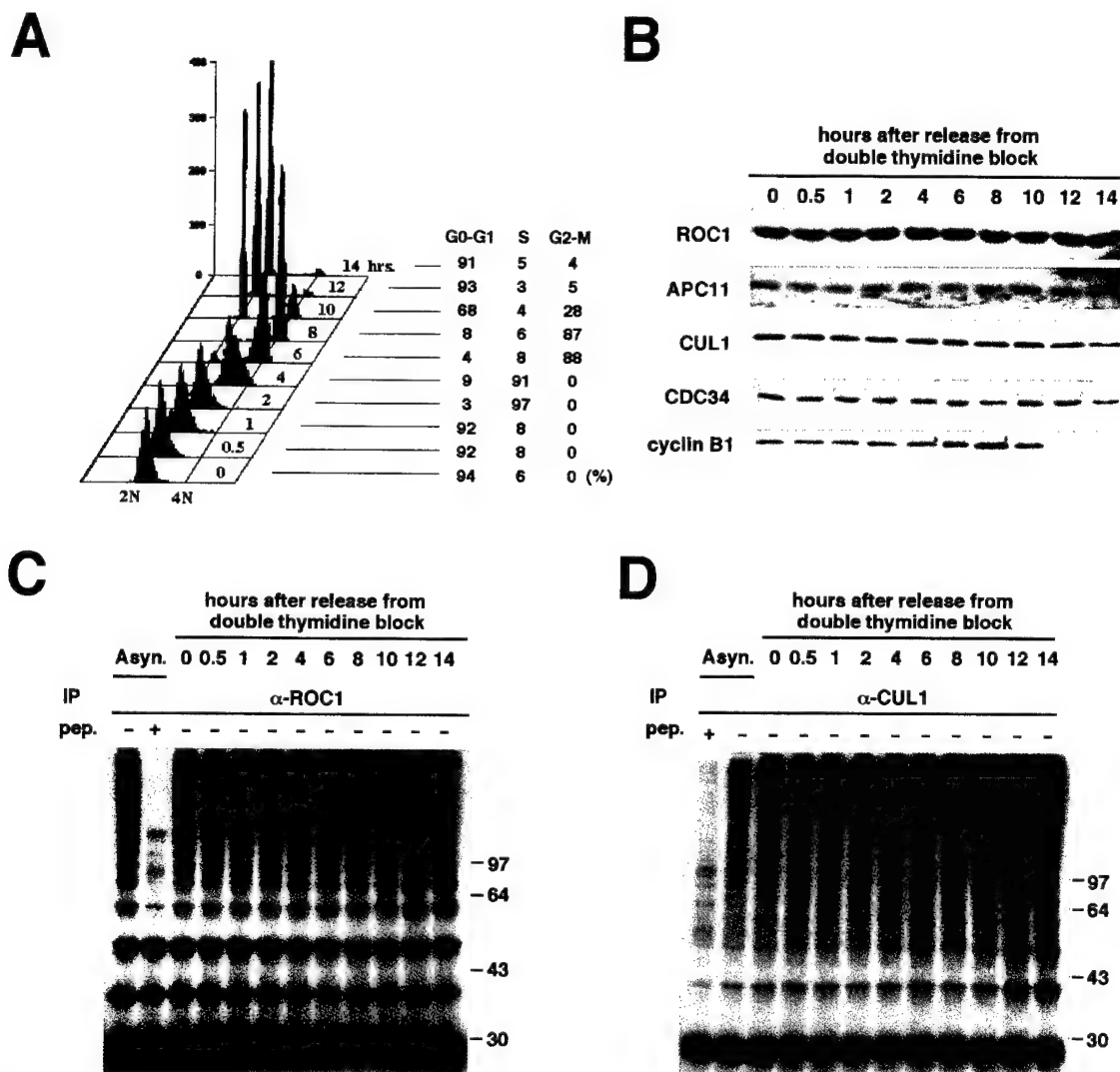


Figure 2 Cell cycle expression of ROC1, ROC2, and APC11. (a) HeLa cells were arrested at the G1/S boundary by a double thymidine block, and progression through the cell cycle after release from the block was monitored by FACS analysis. (b) Total cell lysates were prepared from HeLa cells at the indicated times after release from the G1/S block and 50 μ g of each sample was resolved by SDS-PAGE. The steady state levels of the different proteins were determined by direct immunoblotting. (c, d) Total cell lysates (1 mg) obtained from the cells described in (b) were immunoprecipitated with either anti-ROC1 (c) or anti-CUL1 (d) antibodies in the presence (+) or absence (-) of competing antigen peptide (pep.). ROC1 or CUL1 associated ubiquitin ligase activity was assayed

of both ROC1 and CUL1 activity at 12 and 14 h after release from the thymidine block (enriched for a G1 population), and its significance in promoting G1, if any, is unclear at present. Taken together, these results indicate that the steady state levels of both ROC1 and CUL1 proteins and their associated ubiquitin ligase activities, as determined *in vitro* by a substrate-independent assay, do not significantly oscillate during the cell cycle. It also indicates that the regulation of APC ubiquitin ligase activity, which accumulates during the G2/M transition, remains active in G1 and declines during S and G2, is unlikely carried out by a change in the steady state level of APC11 protein. These results indicate that if substrate dependent ubiquitin ligase activity oscillates during the cycle of exponentially growing cells, it is more likely mediated by targeting signals such as substrate phosphorylation or oscillating expression of targeting proteins.

Endogenous ROC1 and APC11 are stable proteins

To gain insight into the property of ROC/APC11 proteins, we determined the half-life of endogenous ROC1, APC11 and another CDC53/cullin-interacting protein, SKP1 (Figure 3a). All three proteins appear to

be relatively stable. The half-lives of both APC11 and SKP1 are approximately 3 h, and the half-life of ROC1 is more than the 5 h experimental time course. These results indicate that under normal growth conditions, the turnover of endogenous ROC1, APC11, and SKP1 proteins is very slow in exponentially growing cells. This result is consistent with the near absence of fluctuation observed in their steady state levels.

Ectopically expressed ROC1, ROC2 and APC11 are short-lived proteins

Unexpectedly, transfected HA-ROC1 (~26 min), HA-ROC2 (~9 min) and HA-APC11 (~17 min) in Saos2 cells are very short-lived proteins (Figure 3b). As a control, transfected SKP1 remains a relatively stable protein with an estimated half-life similar to that of the endogenous protein (Figure 3b). Transfected CUL1 is also a relatively long-lived protein with an estimated half-life of approximately 3 h. We also determined the half-life of another CUL1 interacting protein, E2 CDC34. The half-life of transfected CDC34 was longer than the 5 h of our experimental time course (Figure 3b), and the endogenous CDC34 was not evidently detected after 4 h of pulse labeling despite the

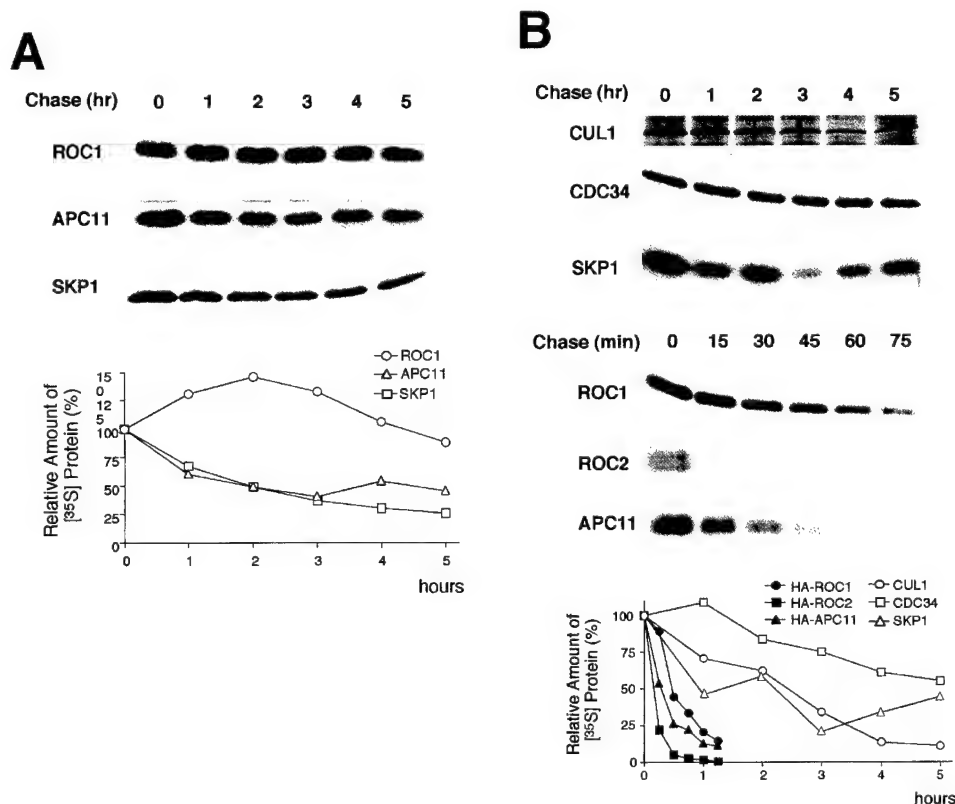


Figure 3 Half-life of ROC-cullin ligase subunits. (a) HeLa cells were pulsed with [³⁵S]-methionine for 30 min and then chased for the indicated lengths of time. Cell lysates were immunoprecipitated with antibodies specific to ROC1, APC11 and SKP1. Immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography. The amount of labeled protein at each time point was quantitated on a PhosphorImager (Molecular Dynamics, ImageQuant software version 3.3), normalized relative to the amount of radiolabeled protein present in cells following the 0-min chase, and plotted against the chase time. (b) Ectopically expressed ROC and APC11 are short-lived proteins. Twenty-four hours after Saos-2 cells were transfected with vectors expressing individual proteins, cells were pulsed with [³⁵S]-methionine for 30 min and then chased for the indicated lengths of time. Cell lysates were precipitated with anti-HA (for ROC1, ROC2 and APC11), anti-CUL1, anti-CDC34 and anti-SKP1 antibodies, resolved by SDS-PAGE and autoradiographed. The amount of labeled protein at each time point was quantitated on a PhosphorImager, normalized relative to the amount of radiolabeled protein in cells chased for 0 h, and plotted against the chase time

abundant amount of CDC34 protein expressed as determined by direct immunoblotting (data not shown and Figure 2b). One possible explanation for the different half-lives of endogenous and ectopically expressed proteins is that ROC family proteins are intrinsically unstable but are protected from degradation *in vivo*. Thus, overexpression may have titrated away protective factor(s), exposing them to degradation signals.

Turnover of ROC proteins are sensitive to proteasome inhibitors

The short half-lives of ROC and APC11 proteins led us to determine if their turnovers are regulated by the proteasome and, hence, ubiquitin-mediated proteolysis. HeLa cells transfected with HA-ROC1 expressing plasmid were treated with LLnL (N-acetyl-leuciny-leuciny-norleucinal) or MG132 (carbobenzoxyl-leuciny-leuciny-leucinal), both inhibitors of 26S proteasome activity. Treatment of transfected cells with either of the proteasome inhibitors, but not solvent DMSO (dimethylsulfoxide), increased the half-life of transfected ROC1 protein from 20 min to around 60 min (Figure 4a). The half-lives of transfected HA-ROC2 (Figure 4b) and HA-APC11 (Figure 4c) proteins were increased from 20 and 30 min to significantly longer than 60 min in treated cells. These results indicate that rapid turnover of ectopically expressed ROC1, ROC2 and APC11 proteins are a result of degradation by an LLnL- and MG132-sensitive pathway, likely by the ubiquitin-mediated 26S proteasome pathway.

Association with cullins stabilize ROC1 and APC11

In searching for the mechanism that confers stability to endogenous ROC/APC11 proteins, we tested the possibility that these proteins are stabilized as a result of association with their cullin partners. Epitope tagged ROC1 (HA-ROC1) or APC11 (HA-APC11) were co-transfected with a control vector or a plasmid expressing CUL1 or APC2 into 293T cells. ROC1-CUL1 and APC11-APC2 complex formation was confirmed by co-immunoprecipitation [confirmatory data not shown, also see (Ohta *et al.*, 1999)]. The half-life of ROC1 in this experiment was increased from 40 min (the half-life of ectopically expressed ROC1 in 293T cells is a little longer than in Saos2 or HeLa cells used for previous experiments) to more than 90 min when co-expressed with cullin 1 (Figure 5a). The half-life of APC11 was increased from 20 to 50 min when co-expressed with APC2 (Figure 5b). These results suggest that association with a cullin protein may explain, at least in part, the different protein stabilities between endogenous and ectopically expressed ROC and APC11 proteins.

Disruption of ROC1-cullin association decreases the stability of ROC1 and abolishes its associated ubiquitin ligase activity

If association with a cullin protects ROC and APC11 from degradation, mutations that disrupt or reduce the binding of ROC proteins with cullins would predict a reduction in ROC stability. To test

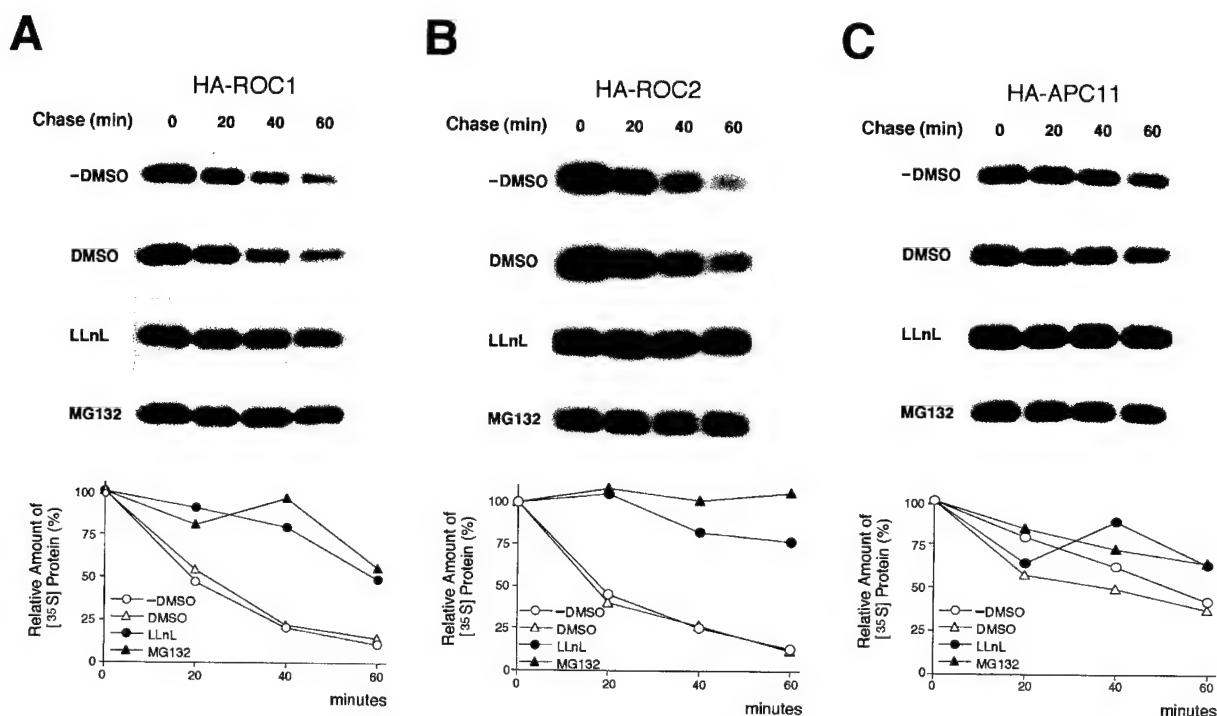


Figure 4 Ectopically expressed ROC family proteins are stabilized by proteasome inhibitors. HeLa cells were transfected with a plasmid expressing HA tagged ROC1, ROC2 and APC11 and 24 h later were treated with LLnL, MG132 or control solvent DMSO for 3 h. Cells were then pulsed with [³⁵S]-methionine for 30 min and chased for the indicated lengths of time. Cell lysates were precipitated with anti-HA antibody, resolved by SDS-PAGE and autoradiographed. The amount of labeled protein at each time point was quantitated on a PhosphorImager, normalized relative to the amount of radiolabeled protein in cells chased for 0 h, and plotted against the chase time

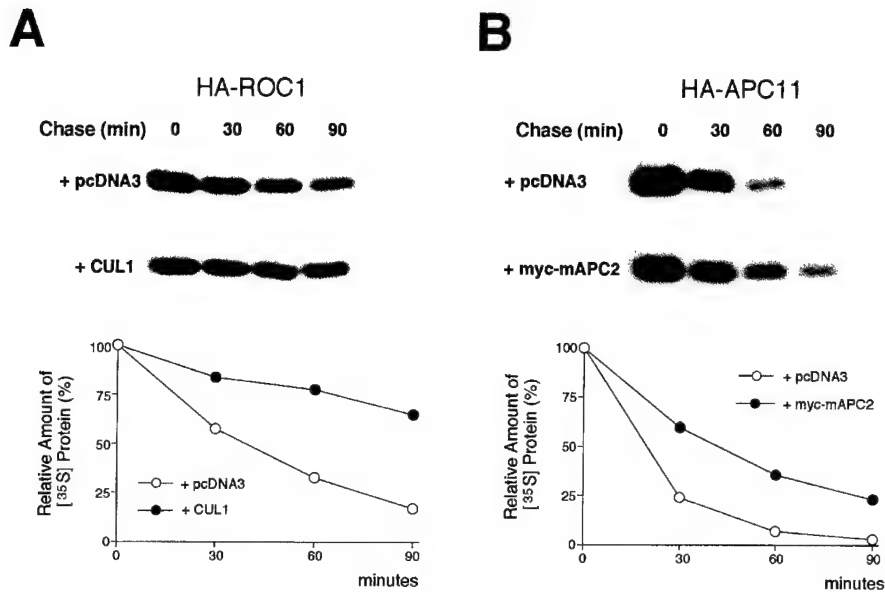


Figure 5 Cullin binding protects ROC and APC11 from degradation. 293T cells were co-transfected with plasmids expressing HA-tagged ROC1 with either control vector pcDNA3 or a CUL-1 expression plasmid (a), or HA-tagged APC11 with either pcDNA3 or an APC2 expression plasmid (b). Twenty-four hours after transfection, cells were pulsed with [³⁵S]-methionine for 30 min and then chased for the indicated lengths of time. Cell lysates were precipitated with anti-HA antibody, resolved by SDS-PAGE and autoradiographed. The amount of labeled protein at each time point was quantitated on a PhosphorImager, normalized relative to the amount of radiolabeled protein in cells chased for 0 h, and plotted against the chase time

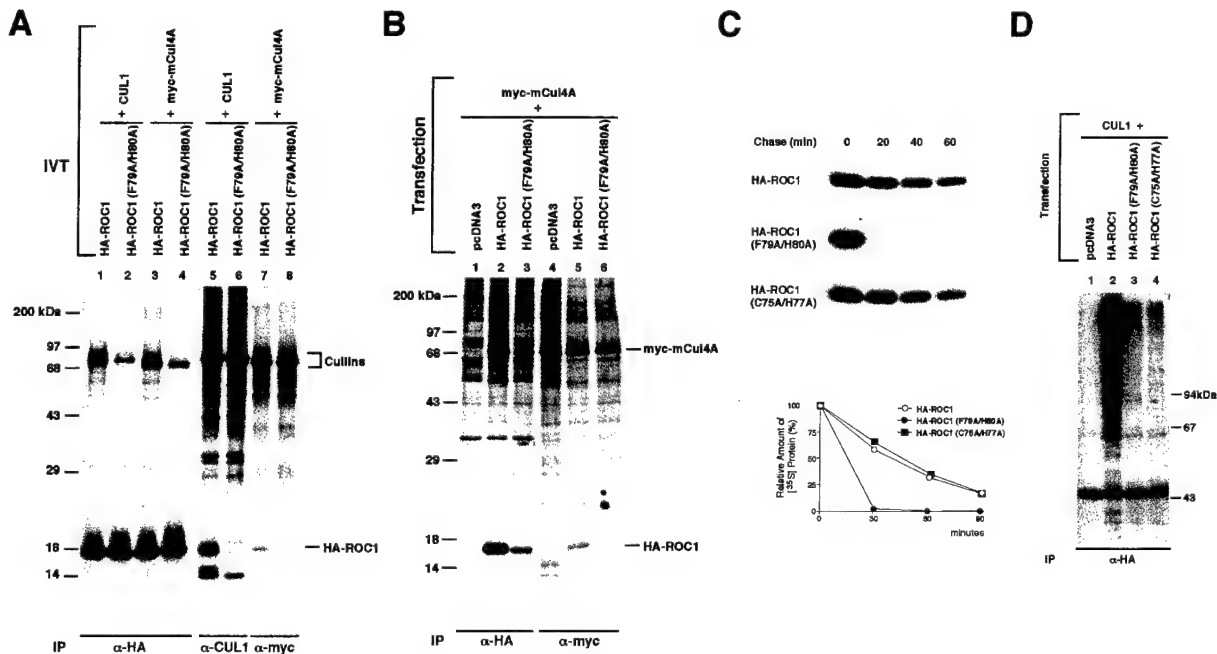


Figure 6 Mutations disrupting ROC1-CUL1 binding diminishes ROC1 stability and ligase activity. (a) *In vitro* binding of ROC1^{F79A/H80A} mutant with cullin proteins. In vitro translated and [³⁵S]-methionine-labeled CUL1 and mouse CUL4A were mixed with wild type or mutant ROC1 for 90 min. Mixtures were immunoprecipitated with indicated antibodies and resolved by SDS-PAGE. (b) *In vivo* binding of ROC1^{F79A/H80A} mutant with CUL4A protein. Myc-tagged CUL4A was co-transfected with wild type or mutant ROC1 into Saos2 cells. Twenty-four hours after transfection, cells were labeled with [³⁵S]-methionine for 2 h. Cell lysates were precipitated with anti-HA or anti-myc antibody, resolved by SDS-PAGE and autoradiographed. (c) 293T cells were transfected with plasmids expressing HA-tagged wild type or mutant ROC1. Twenty-four hours after transfection, cells were pulsed with [³⁵S]-methionine for 30 min and then chased for the indicated lengths of time. Cell lysates were precipitated with anti-HA antibody, resolved by SDS-PAGE, and autoradiographed. The amount of labeled protein at each time point was quantitated on a PhosphorImager, normalized relative to the amount of radiolabeled protein in cells chased for 0 h, and plotted against the chase time (d) F79A/H80A mutation abolishes ROC1-CUL1 ubiquitin ligase activity. 293T cells were co-transfected with a CUL1 expressing plasmid and either vector control or a plasmid expressing wild type or mutant ROC1 as indicated. Thirty-six hours after transfection, cells were lysed and immunoprecipitated with anti-HA antibody, and immunocomplexes were subjected to the ubiquitin ligase activity assay

this possibility, we generated a series of point mutations at residues conserved between members of the ROC/APC11 family from different organisms and searched for those that disrupted the ROC1-cullin association. A double mutation of F79A/H80A substantially reduced the binding of ROC1 with both CUL1 and CUL4A as determined by *in vitro* binding (Figure 6a) and by *in vivo* co-immunoprecipitation (Figure 6b). Ectopically expressed ROC1^{F79A/H80A} was barely detectable after 20 min of chase, significantly shorter than that of the wild type ROC1 transfected in parallel (Figure 6c). Reduced association with cullins and decreased stability is accompanied by a near abolishment of ROC1-associated ubiquitin ligase activity. While the anti-HA immunocomplex derived from cells transfected with CUL1 and wild type ROC1 contain high levels of ubiquitin ligase activity, the anti-HA immunocomplex containing ROC1^{F79A/H80A} exhibited only background levels of activity (Figure 6d). To exclude the possibility that the reduction in ROC1 protein stability caused by the F79A/H80A mutation is not caused by the loss of ubiquitin ligase activity, we determined the half-life of another ROC1 mutant, ROC1^{C75A/H77A}, that abolishes the ubiquitin ligase activity without a detectable effect on its binding with cullins [(Ohta *et al.*, 1999) and Figure 6d]. Unlike the ROC1^{F79A/H80A} mutant, the half-life of transfected HA-ROC1^{C75A/H77A} mutant is almost the same as that of wild type ROC1 (Figure 6c). These results indicate that cullin binding, not retention of the catalytic activity, has a more pronounced effect on the stability of ROC1 protein.

Discussion

Cullin-1/CDC53 represents a multigene family containing three distinct genes in yeast, seven in *C. elegans* and at least six in mammalian cells. In addition, a subunit in the mitotic E3 APC complex, APC2, shares limited sequence similarity with cullins in its C-terminal region. Both CUL1/CDC53 and APC2 function in ubiquitin-mediated protein degradation, implicating other members of the cullin family as components of ubiquitin ligases. Very recently, a RING finger protein, ROC1, Rbx1, or Hrt1, was identified as an essential subunit of CUL1 ubiquitin ligase activity (Ohta *et al.*, 1999; Tan *et al.*, 1999; Kamura *et al.*, 1999; Skowyra *et al.*, 1999; Seol *et al.*, 1999). ROC1 also represents a multigene family containing three closely related proteins, ROC1, ROC2 and APC11 in higher eukaryotes. While ROC1 and ROC2 commonly interact with all cullins, APC11 selectively binds to APC2 (Ohta *et al.*, 1999). How the ROC-cullin family of ubiquitin ligases is regulated is unknown, and this report provides the first evidence that ROC/APC11-cullin/APC2 complex assembly and ROC/APC11 subunit degradation may be one mechanism regulating the activity of this family of ligases.

Our studies have shown that cullin and ROC proteins are present in quiescent cells but are further induced after mitogenic stimulation. These observations suggest that ROC1-CUL1 ubiquitin ligase may function in arresting cells at quiescence, perhaps by

preventing the accumulation of growth promoting proto-oncogene products, and that a higher ROC1-CUL1 ligase activity may be needed for exit from quiescence. How the ROC1-CUL1 is targeted to different sets of substrate is unclear now. The CDC34 protein is undetectable during quiescence and is significantly induced upon introduction of mitogens. Taken together with our previous finding that ROC1-CUL1 complexes may be activated differently by various E2s (Ohta *et al.*, 1999), these observations suggest that CDC34-dependent ROC1-cullin 1 ubiquitin ligase activity may be absent until activated by mitogens. Thus, there may exist *in vivo* three different regulations on targeting the ROC1-CUL1 ligase: the availability of the appropriate E2 to harness the ROC1-CUL1 toward a specific substrate, the availability of substrate-receptor molecules such as F box protein p4^{SKP2} whose level changes in response to viral transformation or oscillates during the cell cycle (Zhang *et al.*, 1995; Marti *et al.*, 1999), and substrate phosphorylation linked to a specific growth condition or a particular phase of the cell cycle [see recently reviewed in Maniatis (1999), Laney and Hochstrasser (1999)]. Through such multi-step regulation, cells may render the ROC1-CUL1 ligase the capacity to ubiquitinate multiple substrate proteins.

The substrate recognition subunits of the ROC1-CDC53 ligase, F-box proteins CDC4 and GRR1, were rapidly degraded by the ubiquitination pathway and protected from degradation by binding with its adaptor SKP1 (Zhou and Howley, 1998; Mathias *et al.*, 1999), suggesting a possible model for regulating the function of ROC1-CUL1 ligase at the level of targeting. The mechanism(s) regulating the assembly and activity of ROC-cullin ligases themselves is not known at present. We have found that while CUL1 and E2 CDC34 are stable proteins, all three members of the ROC family are intrinsically unstable and are rapidly degraded by the proteasome pathway. Hence, of the three core components common to all ROC-cullin ligases, regulation of the level of ROC proteins is most likely to play an active role in ligase complex assembly and activation. Unlike the substrate targeting subunit CDC4 whose overexpression caused cell death (Mathias *et al.*, 1999), overexpression of ROC1 does not seem to inhibit cell growth (Ohta *et al.*, 1999). Decreased levels of ROC1, however, cause cell cycle arrest (Ohta *et al.*, 1999; Skowyra *et al.*, 1999; Seol *et al.*, 1999) indicating that the ROC1 protein must normally be protected from degradation. How ROC family proteins are targeted for degradation is unclear, but binding with a cullin partner seems to confer stability to ROC proteins (Figure 5). Mutations in ROC1 that diminish its binding with CUL1 further decreased its half-life and almost completely abolished its ligase activity (Figure 6). These results suggest that binding with a cullin may stabilize ROC proteins by preventing other factors from binding to and targeting their ubiquitination. It will be important to determine what signal triggers ROC-cullin disassociation and ROC degradation, and whether ROC family proteins are ubiquitinated by ROC-cullin ligases, thereby forming a negative feedback regulatory loop.

Materials and methods

Plasmids

Full length mammalian cullin cDNAs were described in Michel and Xiong (1998). Human ROC1, ROC2, APC11 and mouse APC2 cDNAs were described in Ohta *et al.*, (1999). ROC1 mutations were introduced by site-directed mutagenesis using Quick-Change kit (Stratagene) and verified by DNA sequencing.

Cell culture and Northern analysis

Cell lines, culture conditions, cell transfection procedures and antibodies have been described in (Ohta *et al.* (1999). Procedures for ³⁵S-methionine metabolic labeling, immunoprecipitation, and immunoblotting have been described previously (Jenkins and Xiong, 1995). NHF1 cells were synchronized by serum starvation and stimulation. NHF1 cells were cultured in DMEM containing 15% FBS to 30–40% density, starved in DMEM containing 0.5% FBS for 3 days and stimulated by switching to DMEM containing 15% FBS. For synchronization with a double thymidine block, asynchronously growing HeLa cells were cultured in the presence of 2 mM thymidine for 18 h, washed with PBS, and grown in medium without thymidine for 9 h. Cells were then incubated with 2 mM thymidine for 18 h again and released into fresh medium without thymidine and harvested at indicated times. Progression through the cell cycle was monitored by flow cytometry analysis as previously described (Michel and Xiong, 1998). Procedures for isolation of total RNA and Northern hybridization have been described (Li *et al.*, 1994).

Protein half-life

5 × 10⁵ cells were seeded onto a 6-well dish, transfected after overnight culture with a total of 2 µg of appropriate plasmid DNA, and incubated for 24 h. Cells were pulse labeled with ³⁵S-methionine for 30 min, washed twice with pre-warmed 1 × PBS and chased by culturing in DMEM/10% FBS media for the time indicated in each figure. To determine the regulation of protein turnover by the proteasome, LLnL (final concentration of 100 µM), MG132 (final concentration of 50 µM) or the same volume of DMSO solvent (5 µl/ml culture) was added to the transfected cells during the last 3 h of transfection and during the pulse-chase labeling period.

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Lysates from pulse-chase labeled cells were immunoprecipitated with antibodies as indicated in each figure. The amount of labeled proteins at each time point was quantitated on a PhosphorImager (Molecular Dynamics, ImageQuant software version 3.3), normalized relative to the amount of radiolabeled proteins present in cells following the 0-min chase, and plotted vs chase time.

Ubiquitin ligase activity assay

The procedure for the ROC and cullin associated ubiquitin ligase activity assay was essentially the same as previously described (Ohta *et al.*, 1999; Tan *et al.*, 1999). Purified rabbit E1 ubiquitin activating enzyme was purchased from Affinity Research Products (Exeter, UK). His-tagged mouse CDC34 was purified using nickel beads (QIAGEN). Ub was prepared by subcloning full length Ub as a fusion protein with a 6 × His-tag and protein kinase C recognition site (LRRASV) and purified with nickel beads (Tan *et al.*, 1999). Purified Ub was labeled with ³²P by incubating with γ-³²P-ATP and cAMP kinase (Sigma) at 37°C for 30 min. For ubiquitination assays, either endogenous ROC1 and CUL1 immunocomplexes were immunoprecipitated with anti-ROC1 and anti-CUL1, or transfected HA-tagged ROC1/CUL1 immunocomplexes were immunoprecipitated with anti-HA antibody. Immunocomplexes immobilized on protein A agarose beads were washed and added to an Ub ligation reaction mixture (30 µl) that contained 50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 2 mM NaF, 10 mM Okadaic Acid, 2 mM ATP, 0.6 mM DTT, 0.75 µg ³²P-Ub, 60 ng E1 and 300 ng mouse CDC34. Reactions were incubated at 37°C for 60 min, terminated by adding of 30 µl 2 × Laemmli loading buffer and boiling for 3 min and resolved by SDS-PAGE followed by autoradiography to visualize the ubiquitin ladders.

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MICHEL, J.
DAMD17-98-1-8221

Era of Hope

Department of Defense
Breast Cancer
Research Program
Meeting



June 8-12, 2000
Wilton Atlanta
and Towers
Atlanta, Georgia

PROCEEDINGS
Volume I

**CELL CYCLE REGULATION BY UBIQUITIN MEDIATED PROTEOLYSIS:
ROLES OF CULLIN-ROC UBIQUITIN LIGASES**

Jennifer J. Michel and Dr. Yue Xiong

University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7295

E-mail: jcarlisl@email.unc.edu

Sequential activation and inactivation of cyclin dependent kinases (CDK) regulate eukaryotic cell cycle transitions. The periodicity of CDK activity provides a molecular basis for unidirectional cell cycle progression and is partly controlled by ubiquitin-mediated proteolysis of cyclins and CDK inhibitors. Deregulated expression of G1 CDK inhibitors and G1 cyclins has been directly linked to breast cancer development. The mechanisms regulating the ubiquitination of these two protein families constitute essential components of G1 cell cycle control in mammalian cells, but they are poorly understood at present. The purpose of this study is to better understand these mechanisms in order to identify areas susceptible to therapeutic discovery.

Polyubiquitination of proteins is known to be catalyzed by a cascade of enzymes: E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating). Two issues critical to our understanding of the regulation of protein turnover are how E3 ligases target proteins for ubiquitination and how E3s are regulated themselves. Using cell culture systems and yeast genetic analysis, we have undertaken a study of the multigene Cullin/CDC53 family, proteins that have been implicated in regulated degradation. We have found that Cullins partner with another multigene family of evolutionarily conserved proteins, the ROC/APC11 family, to form complexes that contain E3 ubiquitin ligase activity. We have demonstrated that the CUL1/ROC1 ligase can specifically catalyze the ubiquitination of phosphorylated I κ B α . In yeast we have shown that Roc1p is required for the degradation of a yeast G1 CDK inhibitor, Sic1p, and a G1 cyclin, Cln2p. Furthermore, we have demonstrated functional conservation and specificity among members of the ROC/APC11 family. Our results demonstrate that Cullin/ROC ligases play a role in proteolytic control of G1 cell cycle proteins, and the number of different ROC/APC11 and Cullin combinations potentially implies a wide variety of roles for these ubiquitin ligases. Understanding the mechanisms by which these ligases target cell cycle proteins for degradation could potentially lead to improved diagnostic, prognostic and therapeutic strategies.

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Hope

MICHEL, J.

98-1-8221

International Defense

Breast Cancer

Research Program

June 8-11, 2000

Hilton Atlanta

Atlanta, Georgia

PROGRAM

FINAL AGENDA**SUNDAY, JUNE 11, 2000****SUNDAY, JUNE 11, 2000**

7:00 a.m.	Registration Open	Lobby
7:00 a.m. - 8:00 a.m.	Continental Breakfast Available	Grand Ballroom Foyer
8:00 a.m. - 8:10 a.m.	WELCOME AND MOMENT OF SILENCE * Bettye Green	Grand Ballroom
8:10 a.m. - 8:45 a.m.	KEYNOTE ADDRESS: Exploiting Low Dose Chemotherapy as an Anti-Angiogenic Therapy * Robert S. Kerbel (Introduction by Lynn M. Matrisian)	Grand Ballroom
8:45 a.m. - 10:05 a.m.	PLENARY: THE ROLE OF VASCULATURE IN BREAST CANCER - A MODEL FOR INTERVENTION AND EARLY DETECTION Introduction by Bettye Green Clinical Applications of Angiogenesis Research * Michael S. O'Reilly Non-Invasive Imaging of Gene Expression * James P. Basilion Consumer Perspective * Liz Lostumbo	Grand Ballroom
10:05 a.m. - 10:20 a.m.	BREAK	
10:20 a.m. - Noon	SYMPOSIUM: ANGIOGENESIS AND BREAST CANCER * Chair: Lynn M. Matrisian Matrix Metalloproteinases: Targets for Multiple Stages of Tumor Progression * Lynn M. Matrisian An Overview of Metastasis * Patricia S. Steeg Mechanism of Action of Su6668; A Novel Angiogenesis Inhibitor Which Regresses Established Tumors * A. Douglas Laird Consumer Perspective * Vernal Branch Question and Answer Session	Grand Ballroom
Noon - 1:25 p.m.	SPECIAL LUNCHEON SESSION: DOD BCRP NEW INVESTIGATORS - A LOOK TO THE FUTURE OF BREAST CANCER RESEARCH * Co-chairs: Stacey Young-McCaughan and M. Carolina Hinestroza QUANTITATIVE MODELS FOR CHARACTERIZING BREAST TUMORS AND TREATMENT RESPONSE USING MRI * Savannah C. Partridge POTENTIAL INVOLVEMENT OF THE ALZHEIMER'S DISEASE-ASSOCIATED PRESENILIN-2 GENE IN TUMORIGENESIS * Minh D. To ANTITUMOR IMMUNITY INDUCED BY RECOMBINANT ERBB-2 DNA VACCINES * Shari A. Pilon PERFORMANCE OF A LONG FOCAL LENGTH COLLIMATING OPTIC FOR MAMMOGRAPHIC IMAGING * Cari QUANTITATIVE RADIONUCLIDE IMAGING WITH A COMBINED X-RAY CT-SCINTILLATION CAMERA * H. Roger Tang SYNTHETIC AND IMMUNOLOGICAL STUDY OF A GLYCOPEPTIDE-BASED CANCER VACCINE * Scott Kuduk CELL CYCLE REGULATION BY UBIQUITIN-MEDIATED PROTEOLYSIS: ROLES OF CULLIN ROC UBIQUITIN LIGASES * Jennifer J. Michel	Grand Ballroom West